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Time-Course of Acute Exercise-Induced Endothelial Dysfunction

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelors of Science in Kinesiology and Health Sciences from

The College of William & Mary

By

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Accepted for Honors _____

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Abstract

Regular endurance exercise improves endothelium-dependent vasorelaxation and results in minimized cardiovascular disease (CVD) risk. The effect of acute exercise on the other hand, is more unclear. It has been proposed that the initial stress of acute exercise may impair vascular function prior to the onset of a beneficial adaptive response. In this study, we examined the time course of impairment in endothelial function in rat aortas following an acute, exhaustive bout of treadmill exercise. Along with that, we examined the corresponding magnitude of observed impairment and eNOS expression, SIRT1 expression, and antioxidant capacity (mM). We used 7-9-month-old, male, Wistar rats which were divided up into four groups (n=12/group): sedentary (SED), 6h post-exercise (6h), 24h post-exercise (24h) and 48h post-exercise. Exercise consisted of one bout of exhaustive treadmill exercise lasting between 30-40min. eNOS and SIRT1 expression was determined by Western Blot, antioxidant capacity (mM) was determined by performing a Caymans chemicals assay and endothelium-dependent vasorelaxation was assessed by constructing an acetylcholine dose response curve (10^{-9} - 10^{-5} M) in a wire myograph. eNOS expression demonstrated a significant ($p<0.05$) increase (1.4 fold) following 6h versus SED. SIRT1 expression was significantly ($p<0.05$) increased (1.7 fold) 24h following exercise versus SED but not at 6 or 48h. Maximal vasorelaxation was impaired in 6h ($71.1\pm4.1\%$) and 24h ($74.1\pm4.5\%$) compared to SED ($87.1\pm4.1\%$) and 48h ($80.7\pm4.3\%$). Our EC_{50} measurement further supports our observation of vascular impairment; the 6h group ($3.89e^{-7}$) was significantly ($p<0.05$) greater than SED ($1.34e^{-7}$) and 48h ($1.52e^{-7}$). However, antioxidant capacity was not significantly increased until 48h-post exercise (0.61 ± 0.15 mM) compared to SED (0.14 ± 0.05 mM). These data suggest that acute, exhaustive treadmill exercise can result in impaired endothelial-dependent vasorelaxation 6h post-exercise and returns and possibly improves within 24-48h during which time antioxidant capacity has increased. In addition, the changes in eNOS expression observed do not adequately explain the impaired endothelial-dependent vasorelaxation observed up to 24h post-exercise.

Background

Cardiovascular exercise is a common prescription for the prevention of various diseases associated with aging such as cardiovascular disease, obesity, and diabetes. Specifically, the benefits of regular exercise on cardiovascular disease risk factors include an increase in exercise tolerance, reduction in body weight, reduction in blood pressure, reduction in low density lipoprotein (LDL) and total cholesterol, increase in high density lipoprotein (HDL) cholesterol and increase in insulin sensitivity (Jonathan Myers, 2003). Over the years, these beneficial effects of regular physical activity have been elucidated to the general public, allowing individuals at risk for cardiovascular disease (CVD) to make immediate changes. Recently, it has been observed that there is an effect of aerobic exercise on the functionality of the vascular endothelium (Dimmeler & Zeiher, 2003). The vascular endothelium is succinctly discussed by Cahill & Redmond (2016) as, "...an interface between the blood stream and the vessel wall. The endothelium responds to humoral, neural and especially hemodynamic stimuli and regulates platelet function, inflammatory responses, vascular smooth muscle cell growth and migration, in addition to modulating vascular tone by synthesizing and releasing vasoactive substances" (p. 97). The endothelium is believed to be a critical component in the pathogenesis of atherosclerosis, giving rise to the term endothelial 'dysfunction' which encompasses the risk of the diseases' onset and its subsequent progression (Cahill & Redmond, 2016). The endothelial cells lining the entire vascular system perform vital functions to maintain the systems' integrity, as outlined by Rajendran et al., (2013), "These functions include fluid filtration, such as in the glomeruli of the kidneys, blood vessel tone, hemostasis, neutrophil recruitment, and hormone trafficking" (p.1057). Due to the numerous functions carried out by this organ, any modifications in its functionality have serious side effects on the vascular system as a whole.

Exercise is a mode of physiological stress that acts on the body and while it ultimately benefits the vascular systems' efficiency in the long-term, there is much debate on its beneficial effects on the body in the short term. Essentially, there is a threshold the body must reach in order to adapt to the stress of exercise. Too little exercise, and the body cannot adapt. Too much exercise, and the vascular system is more susceptible to damage. With the commencement of physical activity, there's an increase in free radicals being produced which can lead to an imbalance between reactive oxygen species (ROS) and antioxidants, referred to as oxidative stress (Urso & Clarkson, 2003). How much harm oxidative stress is doing to the body during an

exercise bout is unclear, but most research suggests that persistent oxidative stress ultimately leads to an accumulation of oxidative damage as well as, "...activation of stress-sensitive signaling pathways and development of pathologic conditions such as cardiovascular disease, insulin resistance and metabolic syndrome." (Yavari, Javadi, Mirmiran, & Bahadoran, 2015). These findings have led to speculation on the time-course of resulting endothelial dysfunction and if there can be instantaneous beneficial effects observed on endothelium function. Haram et al., (2005), carried out an experiment examining the time-course of endothelial dysfunction after a single bout of aerobic exercise and six weeks of regular aerobic exercise in rats. They found that following a single bout of exhaustive aerobic exercise, an immediate improvement of endothelium-dependent vasodilation was observed. In other words, there was an immediate improvement in vascular relaxation which is important for reducing the risk of developing various cardiovascular diseases. In contrast, Robinson et al., (2017), observed that acute resistance exercise impairs endothelium-dependent dilation in arterioles in sedentary individuals, but not exercisers. They mentioned in their conclusion that further studies are warranted to identify a mechanistic pathway and its' importance for maintaining vasorelaxation during stress (Durand et al., 2015). Another study done by Dawson et al., (2008) measured changes in endothelium-dependent vasodilation of the brachial and superficial femoral arteries in male non-elite runners before and after a marathon. Their data suggests that prolonged intensive exercise acutely impairs endothelial function, which may potentially increase risk of cardiovascular events. They were not able to identify a specific mechanism responsible for their observations, but hypothesized a high likelihood that the brachial and femoral arteries are exposed to different flow and shear rate patterns during bouts of lower limb exercise, and mentioned previous findings have suggested differential impacts on NO(nitric oxide)-mediated vasorelaxation in active and inactive muscle beds (Dawson et al., 2008). As shown by these contrasting results, a consensus on this topic has not been reached and is continuing to be investigated. To further emphasize the discrepancy seen in the literature, Robinson et al., (2017) clearly states, "Some studies demonstrate that acute aerobic or resistance exercise impairs vascular function, some demonstrate no effect, and some demonstrate an increase in vascular function following acute aerobic or resistance exercise" (n.p.). Their group goes on to explain the possible factors involved that could be responsible for divergent findings such as, participant sex, age and

health/fitness status, varied intensities of the acute exercise stimulus, and assessments being taken at varied time points (Robinson et al., 2017).

The aim of this study was to determine if a single acute bout of exhaustive aerobic exercise would result in a state of temporary vascular dysfunction which would in turn initiate an adaptive response and eventual improvement in vascular health. In addition, we investigated the potential mechanisms involved in this process including endothelial-dependent vasorelaxation, endothelial nitric oxide synthase (eNOS) and SIRT1 expression as well as markers of nitrosative and oxidative stress. The results of this study could inform the public about appropriate exercise prescription as well as potential targets for pharmacologic intervention.

Endothelial Dysfunction.

Endothelial dysfunction is a condition regarded as a precursor for the development of various cardiovascular diseases. Endothelial dysfunction is characterized by a reduction of the bioavailability of vasodilating agents, nitric oxide (NO) in particular, whereas endothelium-derived contracting factors are increased (Bonetti, Lerman, & Lerman, 2003). This contracting and dilating factor imbalance leads to an impairment of endothelium-dependent vasodilation, which represents the functional characteristic of endothelial dysfunction (Bonetti, Lerman, & Lerman, 2003). This condition is specifically associated with hypertension, coronary artery disease, chronic heart failure, peripheral vascular disease, diabetes, chronic kidney failure, and severe viral infections (Rajendran et al., 2013). When the endothelium is functioning normally, it helps regulate blood clotting, the immune response, the volume of fluid and the amount of electrolytes and other substances that pass from the blood into the tissues, and produces dilation or constriction of the blood vessels (Rajendran et al., 2013). Maintaining the health of the innermost layer of the vessel in order to prevent the gradual onset of a more serious pathology is imperative.

Due to a better understanding of the vascular biology of the endothelium, the development of clinical tests to evaluate several of the functional properties of the endothelium have emerged (Deanfield, Halcox, & Rabelink, 2007). The development of noninvasive techniques to study endothelial function has been a notable advance in the evaluation of patients during the long preclinical stage of atherosclerosis, noted by Deanfield et al., (2007). Presently, the most commonly used method involves flow-mediated dilation (FMD) of the brachial artery

using ultrasound imaging (Gokce, 2011). The brachial diameter is first measured at baseline and after an increase in arterial shear stress induced by inflation, then deflation of a sphygmomanometric arm cuff that elicits an increase in blood flow and brachial vasodilation, primarily caused by NO release (Gokce, 2011). This method correlates with endothelial function in the coronary circulation, relates to traditional risk factors, improves with targeted treatment, and predicts risk of future cardiovascular events (Gokce, 2011). The only downside of using this technique is that it does not reveal information concerning what is going on at the cellular level. This is why endothelial dysfunction is typically studied in model organisms such as mice or rats, which allows further analysis to be done on tissues like examining protein expression. The ability to reverse endothelial dysfunction opens a door for therapeutic interventions which could ultimately improve vascular health and reduce the incidence rate of cardiovascular disease.

Endothelial Nitric Oxide Synthase (eNOS).

Nitric oxide (NO) is a powerful vasodilator that serves as an important protector of the cardiovascular system. This vasoactive agent is produced in the endothelial cell from the amino acid L-arginine, then proceeding to diffuse locally to the vascular smooth muscle where it increases intracellular levels of cyclic GMP and ultimately causes vascular relaxation (Gilligan et al., 1994). NO can be released in response to various stimuli, such as acetylcholine, catecholamines, fluid shear stress; and it regulates basal vascular tone (Poveda et al., 1997). Recently, research has examined the role of exercise on NO bioavailability and its resulting effects on vascular function. Intuitively, increased NO bioavailability within the vasculature during a bout of aerobic exercise would yield beneficial effects on vessel function, allowing appropriate dilation to occur due to an increase in blood flow. A study done by Tanaka et al. (2014), was able to validate this intuition and demonstrated that one bout of moderate aerobic exercise improves endothelial function by increasing NO bioavailability. This in turn, can expect to reduce an individual's risk for cardiovascular disease. Yet, it is important to note that this result was related to moderate intensity exercise as opposed to high intensity which touches on the subject of appropriate exercise prescription. NO availability during high intensity exercise is a question currently under debate, but most research suggests a reduction in NO bioavailability due to a decrease in levels of antioxidants and an increase in reactive oxygen species (Goto et al., 2003). This reduction in NO availability ultimately comes down to impairment in NO's secreting

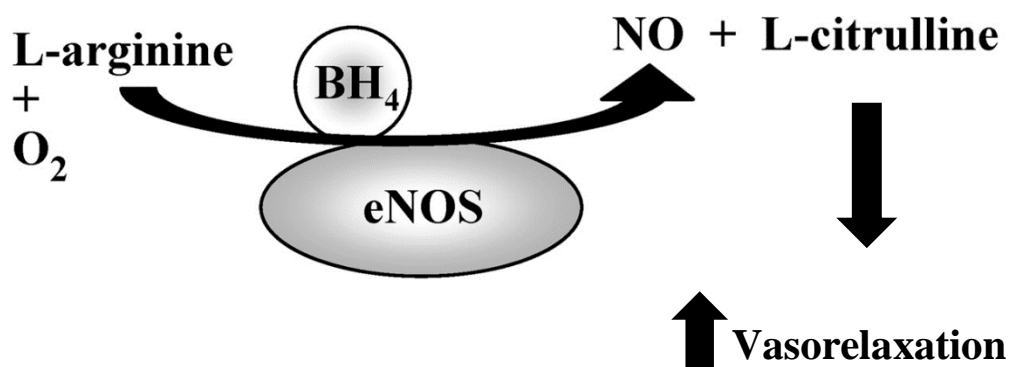
enzyme endothelial nitric oxide synthase, which is essential for the maintenance of a healthy cardiovascular system.

In order for nitric oxide to be released into the vasculature, it requires a functioning secreting enzyme: *endothelial nitric oxide synthase (eNOS)*. eNOS synthesizes NO in a pulsatile manner, and its activity markedly increases when intracellular Ca^{2+} rises in response to fluid shear stress (Förstermann & Sessa, 2012). Fluid shear stress occurs when endothelial cells transduce frictional force from blood flow into biochemical signals that regulate gene expression and cell behavior via specialized mechanisms and pathways (Baeyens, Bandyopadhyay, Coon, Yun, & Schwartz, 2016). A functional eNOS (*'coupled' eNOS*), oxidizes the amino acid L-arginine to L-citrulline and NO. This requires dimerization of the enzyme, the presence of L-arginine, and an essential cofactor (BH_4) which acts as a potent endogenous reducing agent (Förstermann & Münzel, 2006). But, this enzyme can also be transformed into a contributor to oxidative stress, which is commonly referred to as being in an *'uncoupled'* state. Yang & Ming (2006), clearly describe this state, "In eNOS uncoupling, electrons flowing from the reductase domain to the heme are diverted to molecular oxygen rather than to the substrate L-arginine, thereby resulting in production of superoxide instead of NO" (p. 57). There are various biochemical mechanisms suggested to be involved in mediating this uncoupled state, one mechanism to expand on involves BH_4 deficiency.

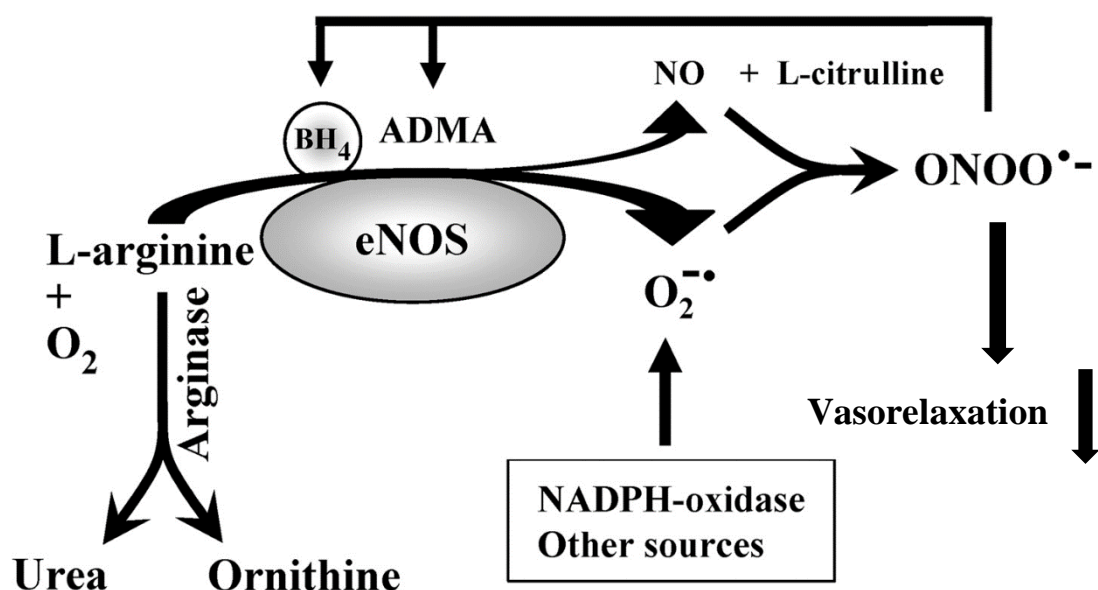
eNOS is a dimeric enzyme consisting of two catalytic domains: a C-terminal reductase domain and an N-terminal oxygenase domain (Kuzkaya, Weissmann, Harrison, & Dikalov, 2003). The reductase domain binds to NADPH, FMN and FAD, while the oxygenase domain binds to a prosthetic heme group, BH_4 , oxygen and L-arginine (Kuzkaya et al., 2003). Kuzkaya et al., (2003) clearly describe BH_4 's critical role in eNOS efficiency, " BH_4 plays a critical role in allowing electron transfer from the prosthetic heme to L-arginine. In the absence of BH_4 , electron flow from the reductase domain to the oxygenase domain is diverted to molecular oxygen rather than to L-arginine" (p. 22546). Which consequently leads to eNOS uncoupling and a significant reduction in nitric oxide (NO) production. Essentially, BH_4 is a critical component of eNOS activity because when BH_4 availability is limiting, eNOS ceases to produce NO and produces the reactive oxygen species: superoxide, which contributes to the progression of vascular disease (Alp & Channon, 2004).

In addition, it has been demonstrated that BH₄ preserves eNOS dimerization which enhances endothelial function (Y.-M. Yang, Huang, Kaley, & Sun, 2009). This is an important note because dimerization is required for eNOS to function effectively. In Yang *et al.*'s (2009) study, they examined eNOS uncoupling and endothelial dysfunction in aged mesenteric vessels of mice. They chose to also look at the dimer stability of eNOS as a contribution to whether or not endothelial dysfunction was present in either young or aged mice. Here they clearly explain the mechanism of eNOS dimerization resulting in NO production, "In an intact eNOS enzyme, the dimer catalyzes flavin-mediated electron transfer from one monomer to the heme of the other monomer. When sufficient substrate L-arginine and cofactor BH₄ are present, intact eNOS dimers couple their heme and O₂ reduction to the synthesis of NO" (p. 1834). Thus, when eNOS is first produced an imbalance of monomer versus dimer can be seen until the entire protein has dimerized or in other words, matured. So, an increase in eNOS expression can be accompanied without an increase in NO availability.

Coupled eNOS



Uncoupled eNOS



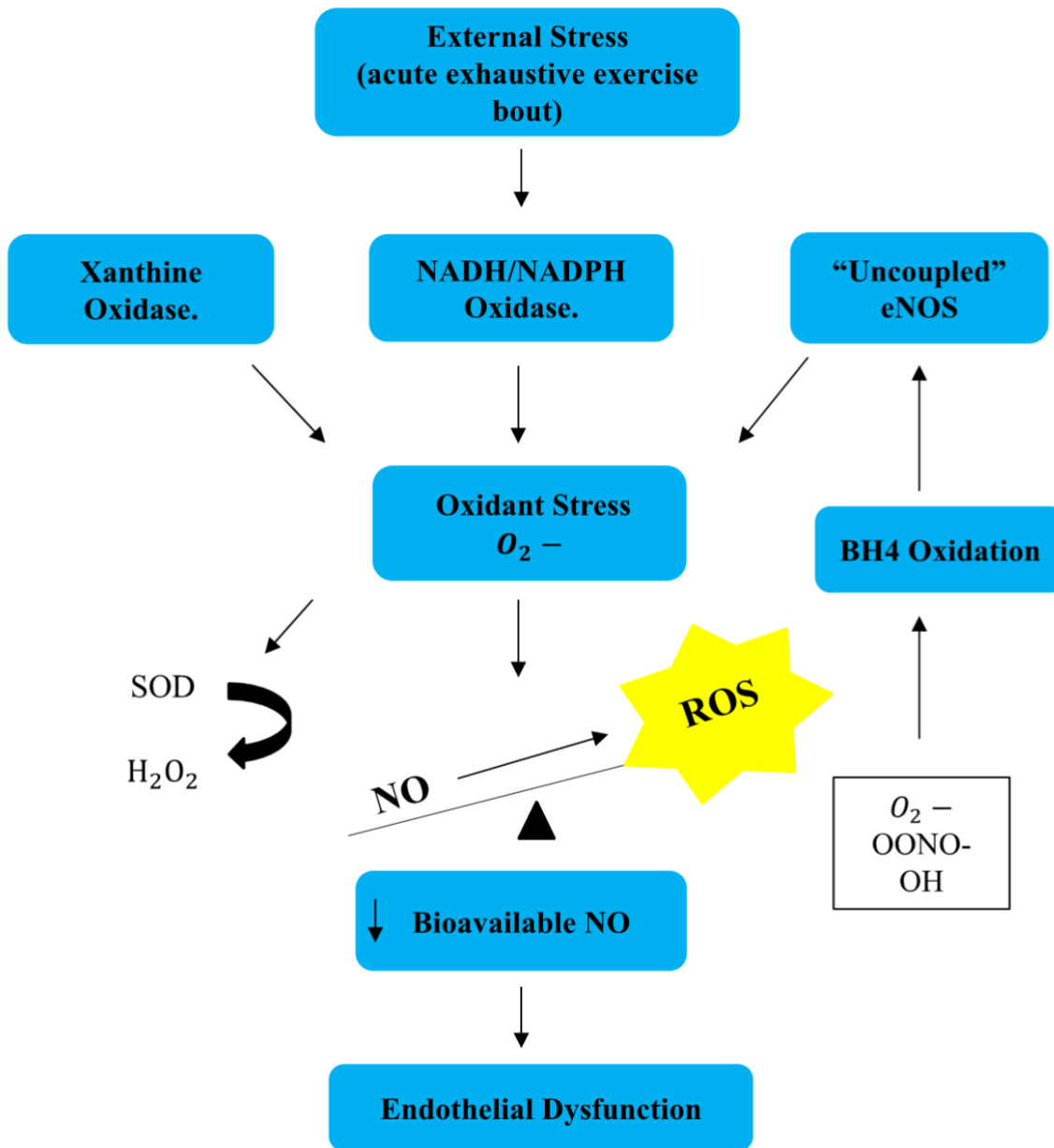
Schematic 1: eNos coupled and uncoupled state, modified from (Yang et al., 2006).

Oxidative Stress.

Oxidative stress refers to an imbalance between free radical production and the endogenous antioxidant defense system, which primarily results in an accumulation of oxidative damage, activation of stress-sensitive signaling pathways and the development of cardiovascular disease, insulin resistance and metabolic syndrome (Yavari et al., 2015). The body fends off free radicals daily due to normal aerobic metabolism, but in particular circumstances like during intensive aerobic exercise, the body is faced with a higher concentration of free radicals to neutralize. When the body is engaged in physical activity, oxidative phosphorylation increases in response to exercise, leading to a subsequent increase in free radicals (Urso & Clarkson, 2003). Increased production of reactive oxygen species (ROS) favors vascular dysfunction by inducing altered vascular permeability and inflammation, along with the loss of vascular regulatory function and an increased expression of inflammatory adhesion molecules (Bielli, Scioli, Mazzaglia, Doldo, & Orlandi, 2015). To reduce the damaging effects of ROS, the body has

evolved both enzymatic and non-enzymatic antioxidant defenses (Scandalios, 2005). Non-enzymatic defenses include compounds with intrinsic antioxidant properties such as vitamin C and E, glutathione and beta-carotene. While purely enzymatic defenses like superoxide dismutase (SOD), catalases (CAT) and peroxidases, work by scavenging superoxide radicals and hydrogen peroxide to neutralize their activity (Scandalios, 2005). Over time, it is believed that with continuous exercise, the body is able to strengthen its' endogenous antioxidant defenses, minimizing oxidative damage.

Along with the discovery of strenuous exercise inducing oxidative damage, has come research involved with investigating the numerous ways to counteract the effects of oxidative stress post-exercise through antioxidant supplementation. Yet, it is known that small concentrations of ROS production works to strengthen the body's endogenous defenses, which allows exercise adaptation to occur. As noted by Pingitore et al (2015), "Regular moderate training appears beneficial for oxidative stress and health. Conversely, acute exercise leads to increased oxidative stress, although this same stimulus is necessary to allow for an upregulation in endogenous antioxidant defenses" (p. 2). A study done by Gomez-Cabrera et al. (2008) observed that vitamin C supplementation seriously decreases improvement in VO₂ max along with running capacity, and went on to conclude, "Physical exercise is a double-edged sword: when practiced strenuously it causes oxidative stress and cell damage; in this case antioxidants should be given. But when practiced in moderation, it increases the expression of antioxidant enzymes and thus should be considered an antioxidant" (p. 130). Results seem to suggest that antioxidant supplementation provides little to no support for helping the body fend off ROS during exercise. So, it must come down to what can be made endogenously. A review by Ji (2002) expands on this notion as well in reference to (Finkel & Holbrook, 2000), explaining how the best way to enhance endogenous antioxidant capacity may actually be oxidative stress itself. This rationale is based on the physiological concept of *hormesis* (Ji, 2002). Ji (2002) defines hormesis as, "a Greek word referring to a sublethal dose of toxin that can increase the tolerance of the organism to withstand higher doses of toxins" (p. 84). Thus, exercising at a high enough intensity that allows for some initial oxidative damage for an appropriate amount of time, will ultimately allow the body to adapt and bolster its' endogenous antioxidant defenses.



Schematic 2: Components involved in oxidative stress leading to endothelial dysfunction.

Modified from Cai & Harrison (2000).

SIRT1.

Sirtuins are a family of NAD⁺ dependent histone/protein deacetylases that have been shown to extend life span in model organisms; yeast and worms (Ferrara et al., 2008). As further described by Ferrara et al. (2008), SIRT1 specifically, is involved in various physiological processes, including gene expression, cell cycle regulation, apoptosis, DNA repair, metabolism, oxidative stress and aging. SIRT1 is best known for its role in the aging process. It has been shown to influence many age related diseases such as metabolic disorders like diabetes, or neurodegenerative diseases like Alzheimer's and Parkinson's, cancer and osteoporosis (Koltai et al., 2010). In the context of aging, SIRT1 is well-known to have a profound effect on the vasculature. Vascular aging is primarily characterized by an up-regulation of NADPH oxidase, oxidative stress and endothelial dysfunction (Zarzuelo et al., 2013). Recent studies have demonstrated the critical role of SIRT1 in the vascular system. It has been reported that SIRT1 promotes endothelial-dependent vasodilation by targeting eNOS for deacetylation, resulting in increased NO production (Ota et al., 2010). Another study conducted by Mattagajasingh et al., (2007) was able to make an interesting connection between caloric restriction (CR) and SIRT1, because CR reduces both systolic and diastolic blood pressure in healthy non-obese individuals. Which may inherently involve the work of SIRT1, but they made the important aside that it would be difficult to attribute the beneficial effect of CR on vascular health solely to SIRT1. Nevertheless, that has opened another avenue of research focusing on SIRT1. In sum, SIRT1 directly acetylates multiple targets (including eNOS), and regulates the transcription of proteins in endothelial cells (Chen, Shentu, Wen, Johnson, & Shyy, 2013). This leads to the functional outcome being increased NO bioavailability, mitochondrial biogenesis, and angiogenesis, but decreased inflammation and oxidative stress (Chen et al., 2013). Thus, increased oxidative stress conditions are associated with impairment of SIRT1 activity, so deacetylation of various targets cannot be done, and endothelial dysfunction arises. These findings clearly indicate the important role SIRT1 plays in mediating endothelium-dependent vasorelaxation through eNOS, and undoubtedly has therapeutic implications against many cardiovascular diseases.

Due to the amount of data reported on SIRT1's role in the vascular system, recent studies have investigated the connection between SIRT1 and exercise. It has been established that an increase in skeletal muscle oxidative capacity during exercise training is important to induce an adaptive response over time. One of the many important components to promoting an adaptive

response to exercise is accelerated mitochondrial biogenesis during training (Gurd et al., 2010). This is because the mitochondria is responsible for oxidizing nutrients to generate ATP which is the primary manner of which increasing energy demands are met during exercise (Drake et al., 2016). A study conducted by Suwa et al. (2008) hypothesized that SIRT1 expression increased after endurance exercise to facilitate metabolic adaptation. And in conclusion, they were able to demonstrate that skeletal muscle SIRT1 expression increases with both acute endurance exercise and endurance training.

SIRT1 has many responsibilities in the body, which is thoroughly outlined by Hwang et al., 2013, “SIRT1 regulates inflammation, aging, calorie restriction/energetics, mitochondrial biogenesis, stress resistance, cellular senescence, endothelial functions, apoptosis/autophagy, and certain circadian rhythms through deacetylation of transcription factors and histones” (p. 12). This family of proteins should seldom be ignored when it comes to the prevention of various age-related diseases, because they have profound implications for not only attenuating cell senescence but improving vascular health.

Methods

Animals.

Experiments were approved by the Institutional Animal Care and Use Committee of The College of William & Mary and complies with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. 7-9-month-old, male Wista rats were divided into four groups (n=12/group): Sedentary (SED), 6h post-exercise (6h), 24h post-exercise (24h), 48h post-exercise (48h) post-exercise.

Exercise Training.

Exercise was done on a motor driven treadmill. Following a preliminary 1-2 week acclimation period to treadmill running, rats were exercised to exhaustion for one day based on their assigned grouping. One acute bout of exhaustive exercise consisted of ~30-40 minutes of

running on the treadmill at 10-15m/min up a 10% grade. A rat was considered 'exhausted' when they sat on the shock grid at the end of the treadmill for more than 3-5 seconds, on more than two occasions.

Vascular Reactivity.

Following the acute exercise bout (6h, 24h or 48h), rats were euthanized with ketamine and xylazine intraperitoneally with a dose of 5-10mg/kg of body weight. Following euthanization, the femoral and aortic arteries were dissected out and cleaned of any extra adhering fat and connective tissue. The following tissues were later dissected out: abdominal fat, liver, plantaris muscle, soleus muscle, EDL (extensor digitorum longus) muscle, and aorta fat. Tissues were immediately frozen with liquid nitrogen and placed for storage in a -80°C freezer.

Rat aortic rings were mounted on a wire myograph in physiological salt solution at 37°C aerated with 95% O₂/5% CO₂. Vascular reactivity was examined by constructing dose response curves to phenylephrine (PE, 10⁻⁹-10⁻³ M), sodium nitroprusside (SNP, 10⁻¹⁰-10⁻⁶ M), and acetylcholine (ACh, 10⁻⁹-10⁻⁵ M). Values are expressed as percent relaxation from 10⁻⁹ M phenylephrine contraction.

Once the aortic arteries were placed in the wire myograph well, they went through an initial 'vessel wake up' procedure in order to reach an optimal resting tension. The aortas sat at ~20mN. Once vessels were stable, they were pre-constricted with the following concentration of phenylephrine: 6.5µl of 10⁻⁴ + 1.3µl of 10⁻³. Once the vessels were pre-constricted and stable, an acetylcholine (ACh) and sodium nitroprusside (SNP) dose response curve was constructed to measure endothelial-dependent vasorelaxation.

Western Blot Analysis.

Aortic tissues were obtained from frozen storage (-80°C) and homogenized in ice-cold lysis buffer using a glass-glass homogenizer. Samples were then centrifuged at 10,000xg for 20 min at 4°C and separated for analysis. After homogenization, approximately 36 µg of each sample was loaded on to a polyacrylamide gel and underwent electrophoresis. They were then transferred to a polyvinylidene fluoride membrane, which was immunoblotted using the Licor Odyssey system according to manufacturer's instructions. The primary antibody used for eNOS expression was mouse, and secondary antibodies used were goat, anti-mouse. Results gathered from immunoblotting were determined by densitometric analysis of the fluorescent bands using the Licor Odyssey Sa imager. The densities for each sample were normalized to a percentage of an aorta standard loaded on each blot and compared to beta-actin as a control.

Antioxidant Capacity Analysis.

Antioxidant capacity was measured using a kit (Cayman Chemicals, #709001) on the aorta tissue samples according to manufacturer's instructions. First the samples were obtained from frozen storage (-80°C) and cells were collected by centrifugation (1,000-2,000 x g for 10 minutes at 4°C). The cell pellet was then homogenized and centrifuged again at 10,000 x g for 15 minutes at 4°C. The last step in pre-assay preparation was removing the supernatant for assay and then stored back in -80°C.

To measure antioxidant capacity the average absorbance (nm) was calculated for each sample and the values were plotted as a function of the final Trolox concentration (mM) standard curve. After the values were plotted on the standard curve, the antioxidant concentration was calculated by using the following equation:

$$\text{Antioxidant (mM)} = \frac{[(\text{Sample average absorbance}) - (y\text{-intercept})]}{\text{Slope}} \times \text{Dilution}$$

Statistical Analysis.

EC₅₀ was determined using a non-linear regression ($P < 0.05$). A two-way ANOVA with a Bonferonni post hoc test ($P < 0.05$) was done for multiple comparisons done against samples. Percent relaxation responses were calculated beginning from a 10^{-9} M constriction of phenylephrine. The present data eliminated the aortas that were non-responsive to acetylcholine-induced relaxation. Animals that died during surgery were also not included in the analysis due to damage done to the endothelium. Results from immunoblotting for eNOS and SIRT1 as well as antioxidant capacity were analyzed using a one-way ANOVA with Dunnet's post-hoc tests ($P < 0.05$).

Results

Aortas contractility in response to phenylephrine remained normal amongst all groups.

Aorta contractility amongst all groups (SED, 6h, 24h, 48h) responded normally to cumulative doses of Phenylephrine (10^{-9} - 10^{-3} M). Their baseline resting tension value was ~20 mN and contracted maximally to 45-50 mN. These results are shown in Figure 1. These results suggest there is no effect on vascular contractility following an acute bout of exhaustive treadmill exercise.

Acetylcholine-induced vasorelaxation is significantly impaired in both 6h and 24h groups compared to 48h and SED.

Endothelial-dependent vasorelaxation was significantly impaired in response to cumulative doses of Acetylcholine (10^{-9} - 10^{-5} M) in the (6h) group and (24h) group compared to the SED and (48h) groups as shown in Figure 2. The vascular relaxation response was measured in % relaxation, so the longer it took for the (6h) and (24h) group to reach a 100% relaxation response indicates impairment on the endothelial level. Acetylcholine was used to measure the relaxation response due to it being a primary mediator of endothelial dependent vasorelaxation.

The EC₅₀ measurement for Acetylcholine is significantly higher in the 6h group.

Table 1 corresponds to the data demonstrated in Figure 2. The EC₅₀ represents the concentration of drug where 50% of its maximal effect is observed, this reflects the mid points of the previous curves (Fig. 1 & 2). This essentially acts as a measurement of sensitivity, in the (6h) group it took significantly ($p < 0.05$) more acetylcholine (3.89×10^{-7}) to elicit half the maximal relaxation response compared to SED ($87.1 \pm 4.1\%$) and 48h (1.52×10^{-7}).

SNP-induced vasorelaxation remained normal amongst all groups.

The sodium nitroprusside (SNP) induced vasorelaxation response remained normal amongst all groups (Fig. 3); they were all able to reach 100% maximal relaxation. SNP works as a NO donor, meaning that the NO bypasses the endothelium and permeates straight to the smooth muscle layer inducing relaxation. These data suggest that there is no short-term impairment in the smooth muscle, the impairment lies in the endothelium.

eNOS expression is significantly increased in 6h group compared to SED.

The Western blot analysis demonstrated significantly ($p < 0.05$) higher expression of eNOS in the (6h) group compared to the SED group (Fig. 4A) by 1.4-fold. Expression is recorded in arbitrary units and normalized against the housekeeping gene used (beta-actin). All samples are referenced to expression in the SED group, which are assigned values of 1. Values of expression are slightly higher in the (24h) and (48h) group but were not statistically significant.

SIRT1 expression is significantly increased in the 24h group compared to SED.

The Western blot analysis demonstrated significantly ($P < 0.05$) higher expression of SIRT1 in the (24h) group compared to SED (Fig 4B) by 1.7 fold. Expression is recorded in arbitrary units and normalized against the housekeeping gene used (beta-actin). All samples are referenced to expression in the SED group, which are assigned values of 1. SIRT1 expression in the (6h) and (48h) group were not significantly different than SED.

Antioxidant capacity is significantly increased in the 48h group compared SED.

Antioxidant capacity (mM) was observed to be significantly ($p < 0.05$) highest in the (48h) group (0.61 ± 0.15 mM) compared to the SED group (0.14 ± 0.05 mM) (Fig. 5). There was a slight decrease in antioxidant capacity in the (6h) group but this result was not significant.

Discussion

The aim of this study was to examine changes in vascular function in rat aortas following an acute, intensive bout of exhaustive aerobic exercise. Specifically, we examined the time course of post-exercise endothelial impairment and the mechanisms that might explain that

impairment. We found that there was significant impairment in the 6h group post exercise (fig. 2) along with an increase in eNOS expression (fig. 4A). In addition, we observed a significant increase in SIRT1 expression 24h post-exercise (fig. 4B) and a significant increase in antioxidant capacity 48h post-exercise (fig. 5). Examining the time-course of exercise-induced endothelial dysfunction provides more insight in regard to vascular health, maintenance and appropriate exercise prescription to minimize cardiovascular disease (CVD) risk. Increasing evidence has found that functional impairment of the endothelium is one of the first signs of the development of atherosclerosis, and without any intervention can ultimately result in the onset of a more serious pathology, CVD (Park & Park, 2015). The endothelium itself is a master regulator of vascular homeostasis; it exerts a great deal of vasoprotective effects such as: vasodilation, suppression of smooth muscle cell growth and inhibition of inflammatory responses, to name a few (Davignon & Ganz, 2004). Furthermore, most of these beneficial effects could not be carried out without nitric oxide, which was discussed previously as a critical endothelium-derived vasodilator (Davignon & Ganz, 2004). Without sufficient production and bioavailability of NO, the protective functions of the endothelium are compromised, which constitutes endothelial dysfunction. One of the most prominent methods to effectively evade this NO imbalance and resulting endothelial dysfunction altogether, is engaging in regular aerobic exercise.

It is well-known that regular participation in aerobic exercise has a favorable effect on many established risk factors of cardiovascular disease (J. Myers, 2003), including lower risk of type 2 diabetes, some forms of cancer and age-adjusted all-cause mortality (Riebe et al., 2015). But, the favorable effects if any, of acute exercise is still unclear. What has often been observed is that it markedly increases oxidative stress, while engaging in long-term moderate aerobic exercise may upregulate antioxidant enzymes that decrease indices of oxidative stress (Laufs et

al., 2005). In light of this, a study previously discussed by Haram et al. (2005), wanted to understand further just how powerful exercise training has emerged to improve endothelial-dependent vasorelaxation and what effects it has on the vasculature in the short-term. Their results indicated that a single bout of acute, intensive exercise improved endothelium-dependent relaxation for about 2 days, with a peak effect after a 12-24h timeframe. These results seemed puzzling based on the known increase in oxidative stress with exercise as well as our own previously unpublished data. The present study demonstrating temporary endothelial dysfunction provides valuable new information in contrast to the findings of Haram et al., (2005) but in agreement with previous studies demonstrating increased oxidative stress (Higashi, Noma, Yoshizumi, & Kihara, 2009). Instead, the data in our study demonstrates significantly decreased endothelium-dependent relaxation 6h and 24h post-exercise (Fig. 2). This is further supported by changes in the EC50 values (Table 1), which demonstrated decreased sensitivity to acetylcholine in the 6h group. The difference between the present study and that of Haram et al., (2005) could be due to differences in methodology. Haram et al., (2005) used female Sprague-Dawley rats while our study used male Wistar rats. The rationale behind not using female rats was to eliminate the chance of significant hormone fluctuations to influence acetylcholine-induced relaxation. Another difference in methodology includes incubating the vessels with the superoxide scavenger superoxide dismutase (SOD) post-exercise. This enzyme is a superoxide scavenger (O_2^-), which ensures an appropriate response to acetylcholine by fending off any potential oxidative damage. Haram et al., (2005) also worked with a different exercise training protocol that consisted of rats running uphill at a 25° grade for 1h and then alternating between 4 mins at an intensity corresponding to 85-90% of maximal oxygen uptake and 2 mins of active

recovery at 65-70%. Our acute bout of training exercise was continuous for approximately 30-40 minutes at a 10° grade.

To examine potential mechanisms involved in the observed endothelial impairment, eNOS expression was analyzed. We hypothesized that in our 6h and 24h group where impaired relaxation was observed, that there would be an increased expression of eNOS. But, the important caveat is that increased expression does not necessarily entail improved vascular function. To analyze this, a western blot analysis was conducted on aorta tissue samples to quantify expression differences across groups (SED, 6h, 24h, 48h) (Fig. 4A). Our results indicated increased expression in the 6h group but not 24h. This data suggests that eNOS was no longer contributing a vasoprotective effect by increasing NO production, but instead contributing to oxidative stress by producing ROS. This state of eNOS is referred to as being uncoupled, as mentioned previously. A study done by Kerr *et. al*, was also able to demonstrate this when examining the hypothesis that decreased NO availability observed was due to excess O_2^- . For this study they used Wistar-Kyoto (WKY) rats and spontaneously hypertensive stroke-prone rats (SHRSP) to serve as models of genetic hypertension. They examined the expression of eNOS mRNA expression and found it to be increased in the SHRSP group, along with increased O_2^- production compared to the WKY. While these results are promising, this relationship between eNOS expression and O_2^- production is not always observed. Another study done by Bauersachs *et. al*, found contrasting results indicating no increase in eNOS expression in spontaneously hypertensive (SHR) rats despite more O_2^- production. Further studies would have to be done to arrive at a more absolute conclusion.

SIRT1 expression was analyzed to investigate its role in observed endothelial impairment. As mentioned previously, research has demonstrated SIRT1 to play a critical role in

vascular health and promote endothelial-dependent vasodilation (Mattagajasingh et al., 2007; Ota et al., 2010). Another western blot analysis was done to quantify expression differences across groups. We found there to be significantly higher expression in the 24h group compared to SED (Fig. 4B). This change in expression along with eNOS, does not sufficiently explain the vascular impairment observed. While both proteins work to control vascular homeostasis, these data suggest a more complex mechanism is responsible for the impaired endothelial-dependent relaxation we observe in the 6h group.

To evaluate antioxidant capacity (mM) across treatment groups an antioxidant assay was done according to Cayman chemicals' manufacturer instructions, and the results were subsequently graphed as depicted in Figure 5. This data suggests antioxidant capacity was significantly increased in the 48h group and seems slightly decreased in the 6h group but was not statistically significant. A study done by Somani *et al.* found results that validate these present findings in the context of differences in antioxidant enzyme activity. They examined the effects of acute and trained exercise on antioxidant enzyme activity in rats and found there to be a significantly larger increase in enzyme activities within the acutely exercised group. They further conclude that due to a higher mitochondria/cytosolic ratio of antioxidant enzymes in the acutely exercised group, this indicates acute exercise contributed to oxidative stress more than trained exercise. This connects to the present data because due to an observed lower level of total antioxidant capacity (mM) in our 6h group post-acute exercise, which indicates that this group experienced increased oxidative stress. This conclusion can be further supported by our findings present in Figure 2, where significant endothelial impairment was shown in response to acetylcholine-induced relaxation in both 6h and 24h groups. We cannot completely assert that the mechanism behind this observed endothelial impairment is purely driven by oxidative stress

and subsequently, decreased antioxidant capacity. But, these results could indicate oxidative stress playing a large role in the impairment we observed.

In conclusion, this study suggests potential dangers that occur with acute, exhaustive aerobic exercise. If one wants to acquire the benefits of reducing CVD risk, it is critical to engage in physical activity on a regular basis, rather than sporadically. These data suggest that at 6h post-acute exercise the peak of negative effects (i.e., impaired endothelial-dependent relaxation, reduced antioxidant capacity, etc.) are present in rat aortas and confirms previous findings in humans of impaired vascular function following intensive exercise such as marathon running. This study could be improved by treating the vessels with the nitric-oxide synthase (eNOS)-inhibiting arginine antagonist, L-arginine methyl ester (L-NAME). Conducting this additional curve would serve to confirm that the impaired endothelial-dependent relaxation observed was due to the augmentation of NO availability and not another vasodilator. Another approach that could improve this study would include treating the vessels with an antioxidant and then examining vascular function post-exercise helping confirm the hypothesis that the observed endothelial dysfunction is due primarily to oxidative stress. Future studies should also seek to determine if there is a genetic basis to this condition by examining genes mediating the vascular response to acute exercise. This data could provide further insight regarding endothelial dysfunction at the molecular level which could lead to the development of more effective therapeutic interventions and further reduce CVD risk and mortality.

Figures.

Fig. 1

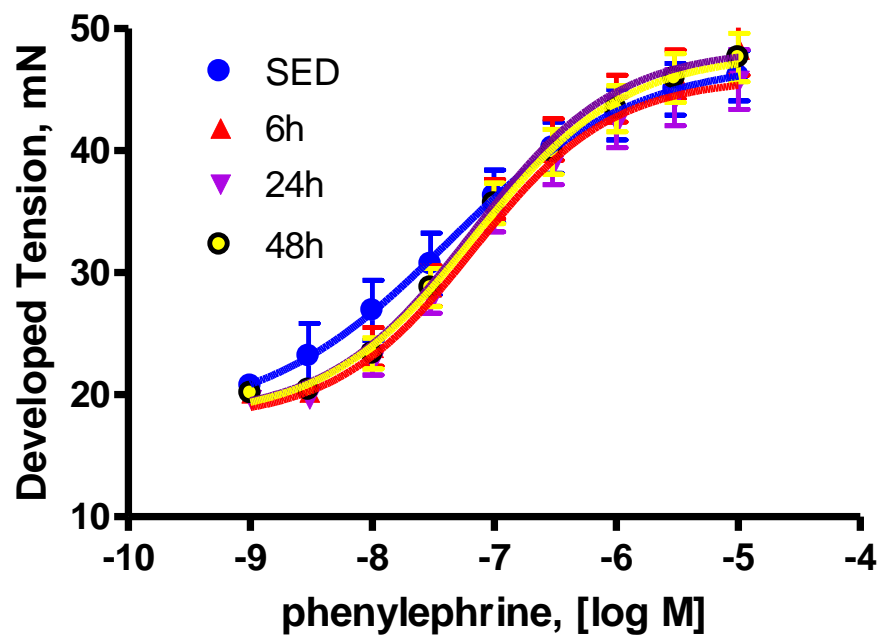


Fig. 2

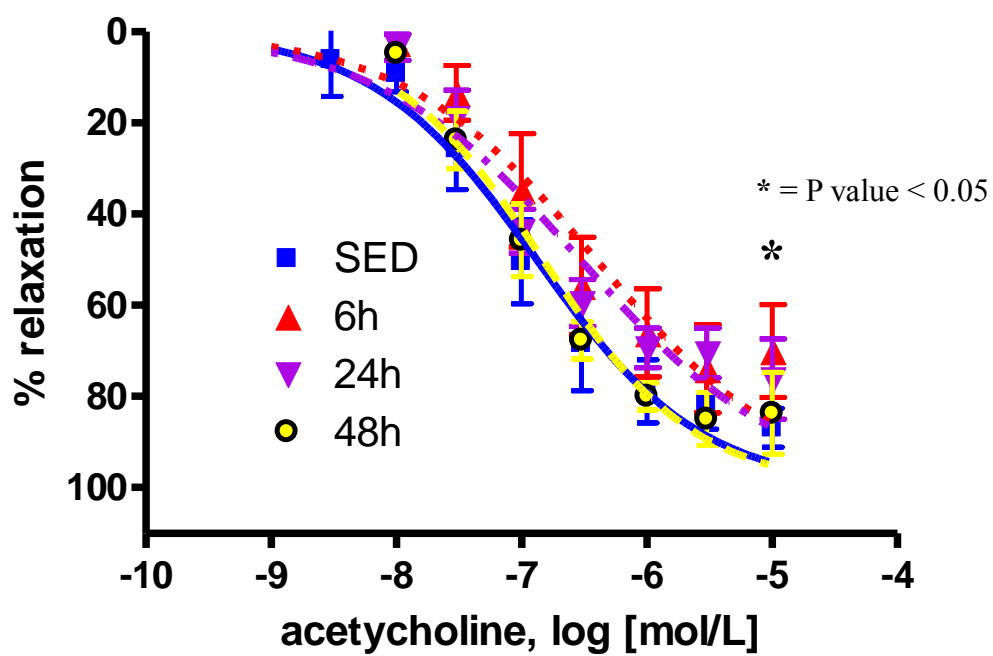


Fig. 3

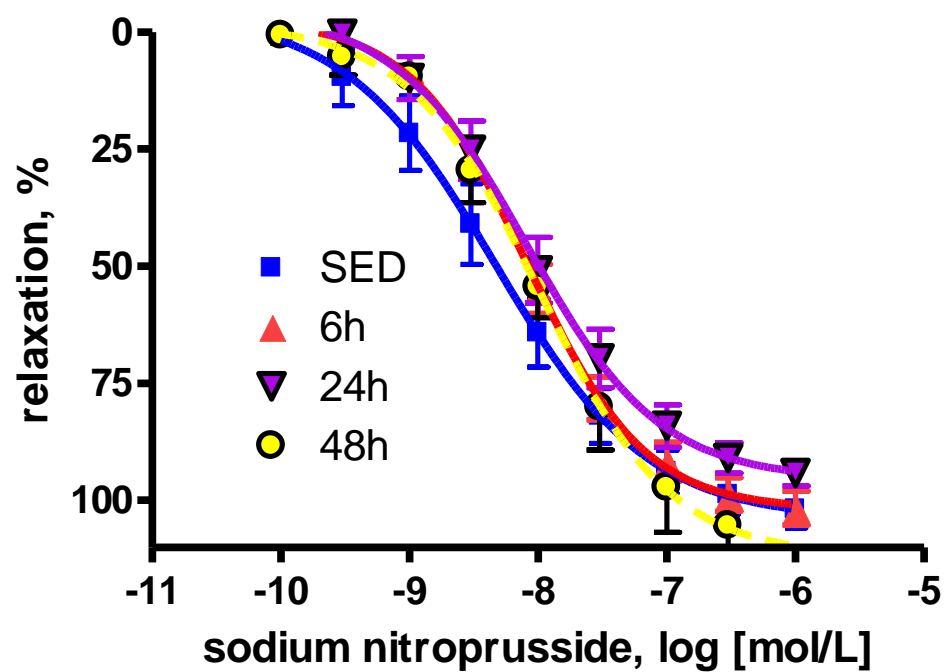
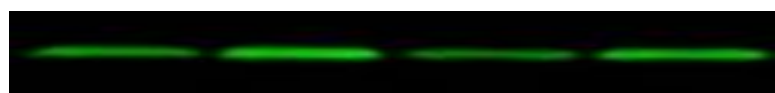
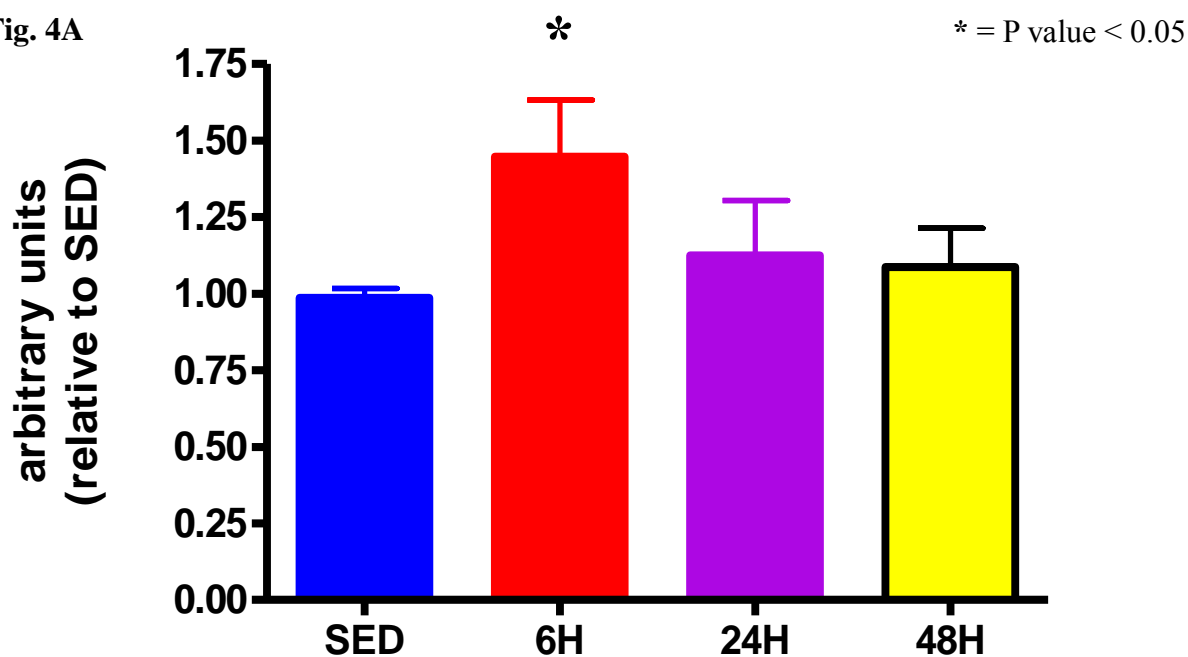


Fig. 4A



eNOS



Beta-Actin

Fig. 4B

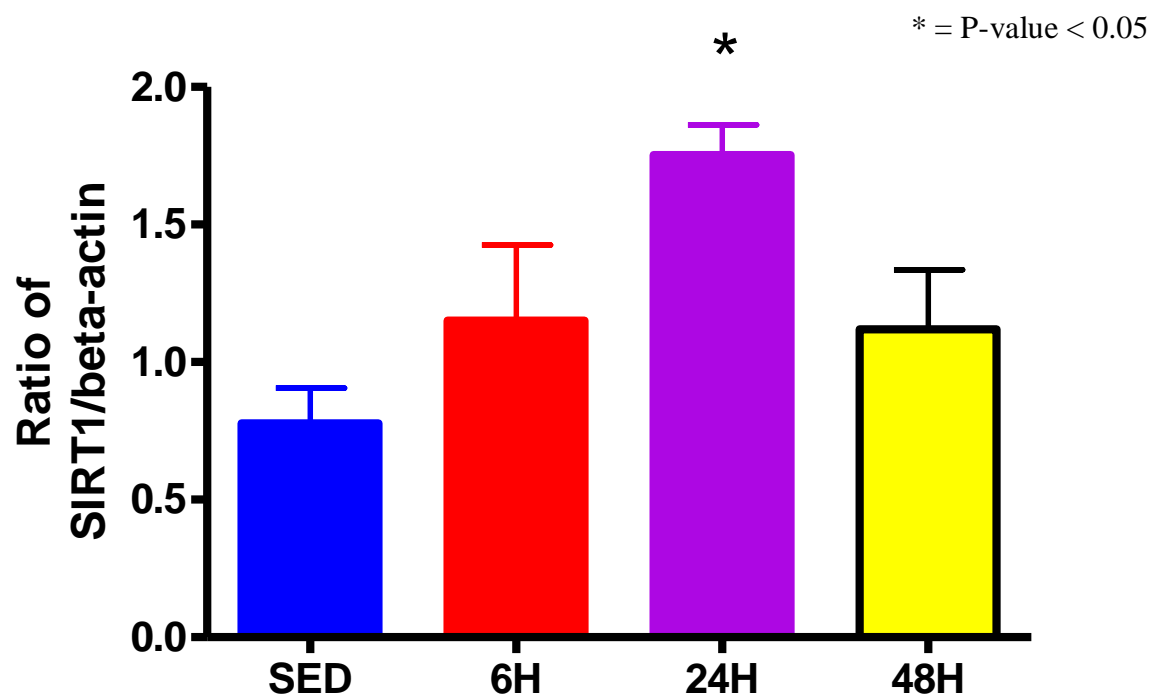
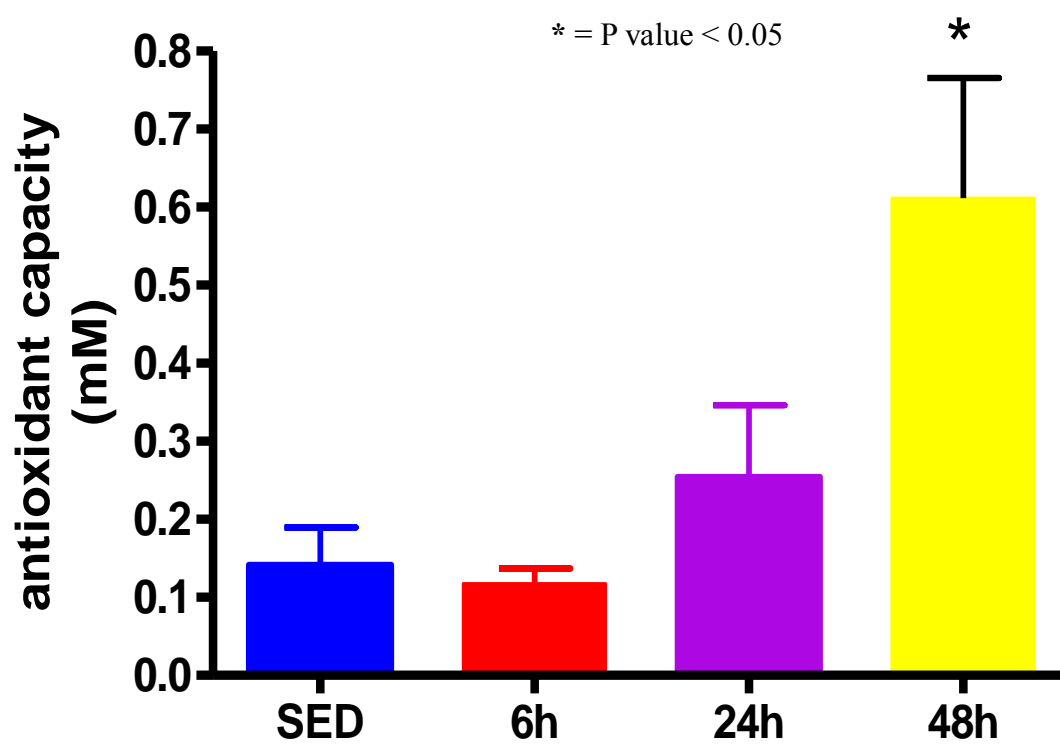


Fig. 5



Tables.

Table 1. Measure of the concentration of Acetylcholine that gives half the maximal relaxation response in rat aortas 6, 24 and 48h post-exercise. EC_{50} for 6h ($3.89e^{-7}$) is significantly greater than SED ($1.34e^{-7}$) and 48h ($1.52e^{-7}$).

(* = P-value < 0.05).

	SED	6 H	24 H	48 H
EC_{50} mM ACH	$1.317e^{-0.007}$	$3.925e^{-0.007*}$	$2.924e^{-0.007}$	$1.474e^{-0.007}$

Figure Captions.

Figure 1: Phenylephrine-induced vasoconstriction in rat aortas across groups (6h, 24h, 48h and SED). The graph depicts the absolute change in tension from baseline tension (mN) following cumulative doses of Phenylephrine (10^{-10} - 10^{-4} M). Data represents no significant changes in response to constriction stimulus post-exercise.

Figure 2: Acetylcholine-induced relaxation in rat aortas across groups (6h, 24h, 48h and SED) following cumulative doses of Ach (10^{-9} - 10^{-5} M). Data is represented as percent relaxation from pre-constriction back to baseline tension (mN). Maximal vasorelaxation was significantly ($p < 0.05$) impaired in the 6h ($71.1 \pm 4.1\%$) and 24h ($74.1 \pm 4.5\%$) compared to SED ($87.1 \pm 4.1\%$) and 48h ($80.7 \pm 4.3\%$).

Figure 3: Sodium Nitroprusside-induced relaxation in rat aortas across groups (6h, 24h, 48h and SED). Endothelial-dependent relaxation was examined after cumulative doses of SNP (10^{-11} - 10^{-5} M). Data is represented as percent relaxation from pre-constriction back to baseline tension (mN). No significant changes observed in response to SNP-induced relaxation.

Figure 4A: eNOS expression relative to Beta-Actin in rat aortas across groups (6h, 24h, 48h and SED). eNOS expression was significantly ($p < 0.05$) increased in the 6h group (1.4 fold)

compared to SED. Data is represented as arbitrary units relative to the SED group which is assigned a value of 1.

Figure 4B: SIRT1 expression relative to Beta-Actin in rat aortas across groups (6h, 24h, 48h and SED). SIRT1 expression was significantly ($p < 0.05$) increased in the 24h group compared to SED. Data is represented as arbitrary units relative to the SED group which is assigned a value of 1.

Figure 5: Antioxidant capacity (mM) in rat aortas across groups (6h, 24h, 48h and SED). Capacity was significantly ($p < 0.05$) increased in the 48h group (0.61 ± 0.15 mM) compared to the SED group (0.14 ± 0.05 mM). Antioxidant capacity was calculated using the following formula: Antioxidant (mM) = $\frac{[(\text{Sample average absorbance}) - (y\text{-intercept})]}{\text{Slope}}$ x Dilution

Schematic 1: Depicts the two redox-dependent states of eNOS: ‘coupled’ vs ‘uncoupled’. In healthy conditions, eNOS is ‘coupled’ with an optimal concentration of the co-factor tetrahydrobiopterin (BH_4), and NO is produced from L-arginine and O_2 as substrates -- inducing increased vasorelaxation. When eNOS is uncoupled, a decrease in endothelial BH_4 production can lead to the subsequent increase of an endogenous eNOS inhibitor, asymmetric dimethylarginine (ADMA). And, an increase in arginase activity which functions to metabolizes L-arginine into urea and ornithine, is observed. Superoxide anion (O_2^-) is produced from this uncoupled state and reacts with NO to generate a more potent oxidant – peroxynitrite (ONOO^-), leading to decreased vasorelaxation and the onset of endothelial dysfunction (Z. Yang & Ming, 2006).

*NADPH-oxidase: one of many oxidative enzymes that contribute to the production of ROS.

Schematic 2: Depicts the various components involved that contribute to oxidative stress and ultimately endothelial dysfunction. Xanthine oxidase and NADH/NADPH oxidases are both families of enzymes that generate ROS and contribute to oxidative stress. Uncoupled eNOS also contributes to oxidative stress and the formation of ROS as discussed previously. ROS listed include: Superoxide anion (O_2^-), Peroxynitrite (OONO^-) and Hydroxide (OH^-). Superoxide

dismutase (SOD) works to neutralize O_2^- to form a more stable ion – hydrogen peroxide (H_2O_2), which combats oxidative stress (Cai & Harrison, 2000).

Appendix.**Abbreviations**

CVD: Cardiovascular disease

ROS: Reactive oxygen species

NO: Nitric oxide

eNOS: Endothelial Nitric Oxide Synthase

FMD: Flow mediated dilation

Cyclic GMP: Cyclic guanosine monophosphate

BH4: Tetrahydrobiopterin

NADPH: Nicotinamide adenosine dinucleotide phosphate

NAD⁺: Nicotinamide adenine dinucleotide

FMN: Flavin mononucleotide

FAD: Flavin adenine dinucleotide

SOD: Superoxide dismutase

CAT: Catalase

CR: Caloric restriction

Ach: Acetylcholine

SNP: Sodium nitroprusside

EC50: Concentration of drug that elicits half-maximal response

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